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Dynamics of the distribution of cyclic AMP-dependent protein kinase in living cells

(nuclear translocation/intracellular distribution/catalytic and type I regulatory subunits)

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ABSTRACT **The intracellular distribution of regulatory molecules may provide a mechanism for controlling gene expression. The subcellular location of cAMP-dependent protein kinase was analyzed in living cells by microinjection of regulatory and catalytic subunits labeled with fluorescein. Following microinjection, type I holoenzyme was found in the cytoplasm and remained there for up to 4 hr. Upon dissociation of holoenzyme with 8-bromo-cAMP, free catalytic subunit appeared in the nucleus while regulatory subunit remained in the cytoplasm. Similarly, purified catalytic subunit was transported to the nucleus in the absence of elevated intracellular cAMP following its introduction into the cytoplasm. Translocation to the nucleus was apparent within 10 min and persisted for at least 2 hr. In contrast, purified regulatory subunit, like holoenzyme, was maintained in the cytoplasm. These results suggest that one function of the type I regulatory subunit is to serve as a cytoplasmic anchor, sequestering the catalytic subunit in the cytoplasm until holoenzyme dissociates in response to increased cAMP.**

Cells can respond to extracellular signals through the action of cell surface receptors and second-messenger pathways. One of the best characterized second-messenger systems in eukaryotic cells is the cAMP-mediated system that leads to the activation of cAMP-dependent protein kinase, cAPK (1). In unstimulated cells, cAPK exists predominantly as an inactive holoenzyme consisting of two regulatory subunits and two catalytic subunits (2). Following an increase in intracellular cAMP, the regulatory subunits bind cAMP, thus causing dissociation of the holoenzyme complex and the production of active monomeric catalytic subunits.

At least two types of holoenzymes, types I and II, exist in most cells. The holoenzyme classification is based on the regulatory subunits, also referred to as type I and type II (3–5). Type II holoenzymes are autophosphorylated on the regulatory subunit (3), whereas type I holoenzymes have a high-affinity binding site for MgATP (4). The amount of cAPK I and II varies between different tissues (6), and the two types of holoenzyme partition differentially between soluble and particulate fractions in the same cell (7, 8). The significance of the different types of regulatory subunits is unclear, but it has been proposed that the regulatory subunit functions in processes other than the inhibition of the catalytic subunit. For example, the type II regulatory subunit binds tightly to other proteins such as microtubule-associated protein 2 (9, 10) and a 150-kDa protein in brain (11). While both the catalytic and regulatory subunits of cAPK have been postulated to play a role in the transcriptional regulation of cAMP-responsive genes (12, 13), more recent studies indicate that the catalytic subunit is both necessary and sufficient for this response (14, 15, 36, 37). Although it seems clear that phosphorylation of key substrates by the catalytic subunit plays a critical role in the transcriptional response to cAMP, it remains uncertain whether the regulatory subunits have functions other than inhibiting the catalytic subunit.

The catalytic subunit of cAPK phosphorylates a wide variety of proteins *in vitro*. It has been proposed that the cellular location of the catalytic subunit may be responsible for determining its physiological substrates *in vivo*. Numerous studies have examined the cellular localization of cAPK subunits, but the results are frequently inconsistent (for a review, see ref. 16; also see refs. 17–22). Subcellular fractionation experiments are hindered by the potential relocation of the catalytic subunit during homogenization, and immunocytochemistry by the inability of the antibodies to discriminate between free catalytic subunits and those present as part of a holoenzyme complex. Here we present an alternative approach in which fluorescently labeled proteins were introduced directly into living cells by microinjection. In this way it was possible to follow the fate of individual subunits or holoenzyme independently. Because microinjected catalytic subunit can function in living cells with respect to both activation of gene expression (14, 15) and altered cytoskeletal structures (23), it is likely that the injected protein is appropriately recognized by the cell.

MATERIALS AND METHODS

Preparation of Fluoresceinated Enzyme Subunits and Holoenzyme. Catalytic subunit was purified from porcine heart (24) or *Escherichia coli* (25, 26) as described. Lysine residues were labeled with fluorescein-5-isothiocyanate (FITC) in 50 mM $NaHCO_3$, pH 8.0/8 mM $MgCl_2$/5 mM ATP at 20°C. A 35-fold molar excess of FITC (128 mM) was used. The reactions were quenched after 20 min by adding a 100-fold excess of 2-mercaptoethanol over FITC. The samples were dialyzed against 5 mM sodium phosphate, pH 7.3/100 mM KCl and filter-sterilized. Bovine type I regulatory subunit was purified as described (27) and labeled similarly to catalytic subunits, except that ATP was omitted and 50 mol of FITC was used per mol of regulatory subunit dimer. Holoenzyme formation was in 25 mM potassium phosphate, pH 6.5–6.8/100 μM ATP/500 μM $MgCl_2$/5% glycerol/5 mM 2-mercaptoethanol. FITC-labeled catalytic subunit was dialyzed with a 1.4-fold excess of regulatory subunit, and FITC-labeled regulatory subunit with a 1.4-fold excess of catalytic subunit, for 1 day at 4°C. Holoenzyme formation was confirmed by spectrophotometric assay in the presence and absence of cAMP and by gel electrophoresis.

Cell Culture and Microinjection. Rat-2 fibroblasts containing a stably transfected cAMP-inducible marker gene (28) were propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units of penicillin per ml, 100 μg of streptomycin sulfate per

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Abbreviations: Br^8cAMP, 8-bromo-cAMP; cAPK, cAMP-dependent protein kinase; FITC, fluorescein-5-isothiocyanate.

ml, and 200 μg of G418 per ml. For microinjection, the cells were plated on scored coverslips and incubated for 24 hr. On the day of injection the coverslips were transferred to 35-mm tissue culture dishes containing growth medium without G418. Proteins were microinjected in 5 mM sodium phosphate, pH 7.3/100 mM KCl at concentrations ranging from 8 to 125 μM. This was diluted 20- to 50-fold by injection; therefore microinjected cells contained from 0.16 to 6 μM injected protein. As it is estimated that cellular levels of cAPK are 0.2–2.0 μM (1), injected cells contained anywhere from endogenous cAPK levels to levels 30 times greater. Proteins were introduced into the cell cytoplasm. At various times following injection, the cells were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 5 min at 22°C and mounted in PBS containing 15% Gelvatol (polyvinyl alcohol), 33% glycerol, and 0.1% NaN_3. The cells were observed and photographed with a Zeiss Axiophot fluorescence microscope under a ×63 (1.4 numerical aperture) oil-immersion lens. Phase-contrast photographs were made with Kodak Tech pan film and fluorescence photographs with Kodak T-Max film. In some cases, the cells were observed and photographed in PBS without prior fixation, under a ×40 water-immersion lens.

RESULTS

Free Catalytic Subunit Is Transported to the Nucleus While Type I Regulatory Subunit Is Maintained in the Cytoplasm. Porcine heart catalytic subunit and bovine type I regulatory subunit were labeled with FITC. Labeled catalytic subunits retained >95% of their initial phosphotransferase activity measured spectrophotometrically (29) (Table 1). The labeled catalytic and type I regulatory subunits retained the ability to reassociate as holoenzyme and dissociate following addition of cAMP (Table 1). The catalytic subunit of cAPK was transported to the cell nucleus following its introduction into the cytoplasm by microinjection. When cells were analyzed immediately after injection, the catalytic subunit was concentrated in a perinuclear location with two distinctly staining structures, possibly centrosomes, visible on either side of the nucleus (Fig. 1). Nuclear staining was apparent within 10 min and maintained for 2 hr. Longer times were associated with cell rounding and an inability to monitor the location of the injected protein. Identical results were obtained in Rat-1 and BALB/c mouse 3T3 cells (data not shown). Catalytic subunit prepared from porcine heart is subject to numerous post-translational modifications, including N-myristoylation (30), which potentially could be important for nuclear translocation. In contrast, catalytic subunit expressed in *E. coli* is not myristoylated. To determine the importance of N-myristoylation for nuclear transport, catalytic subunit purified from porcine heart (Fig. 1) and recombinant murine catalytic subunit (Fig. 2) were injected into cells. Both preparations exhibited strong nuclear fluorescence, suggesting that myristoylation is not obligatory for nuclear transport. Although protein N-myristoylation is thought to occur cotranslationally, it was formally possible that the recombinant catalytic subunit might be a substrate for myristoylation following injection into mammalian cells. To test this hypothesis, recombinant protein containing a mutation sufficient to abolish *in vivo* myristoylation (25) was injected. The non-myristoylated subunit was transported to the nucleus (Fig. 2*b*). To determine the specificity of the nuclear localization of the catalytic subunit, a fluorescently labeled protein having a similar molecular weight, chicken ovalbumin, was injected. Unlike catalytic subunit, ovalbumin was found predominantly in the cytoplasm and within or over the nucleus (Fig. 2*c*). However, the intense nuclear labeling obtained with the catalytic subunit was not observed with ovalbumin. In contrast to the nuclear localization of the catalytic subunit, type I regulatory subunit bound to cAMP was found mainly in the cytoplasm, although its distribution was more selective than that of ovalbumin (Fig. 2*d*). This pattern was strikingly different from that observed with the catalytic subunit, where virtually every injected cell exhibited strong nuclear labeling.

Table 1. Phosphotransferase activity of FITC-labeled catalytic subunits

Sample	Specific activity, μmol/min per mg	
	Before labeling	After labeling
Catalytic subunit		
Porcine heart	17.5	17.4
Recombinant	20.9	20.3
Mutant	22.1	21.8
	− cAMP	+ cAMP
Holoenzyme*		
Exp. 1	0.74	19.6
Exp. 2	1.60	20.1

*FITC-labeled catalytic subunit and type I regulatory subunit.

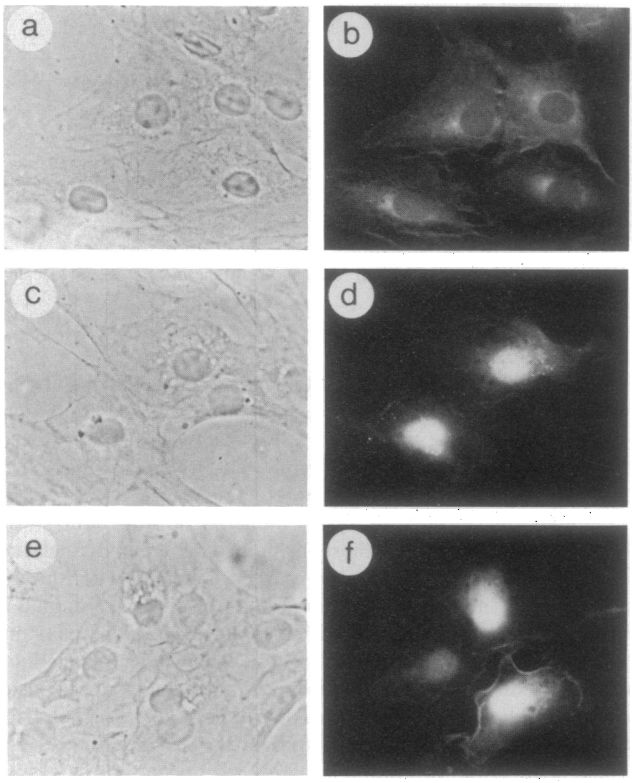

FIG. 1. Free catalytic subunit is transported to the nucleus following introduction into the cytoplasm. Rat fibroblasts were plated on coverslips and grown to 50% confluence. Logarithmically growing cells were injected with fluorescently labeled purified porcine catalytic subunit and fixed immediately (*a* and *b*) or after 30 min (*c* and *d*) or 60 min (*e* and *f*). Phase-contrast photomicrographs are shown on the left and fluorescence photomicrographs on the right. For reference, the nuclei are ≈10 μm in diameter. (×450.)

Type I Holoenzyme Is Cytoplasmic. Since cAPK exists in both holoenzyme and dissociated subunit forms, the distribution of reconstituted holoenzyme containing fluorescently labeled catalytic or regulatory subunits was examined. In either case, the injected holoenzyme was found in the cytoplasmic compartment (Fig. 3 *a* and *c*). Upon dissociation of the holoenzyme complex *in vitro* with a 5-fold molar excess of Br^8cAMP prior to injection or by treating the injected cells with 1 mM Br^8cAMP, significant differences in nuclear staining were observed between holoenzyme preparations containing labeled catalytic subunits and those containing

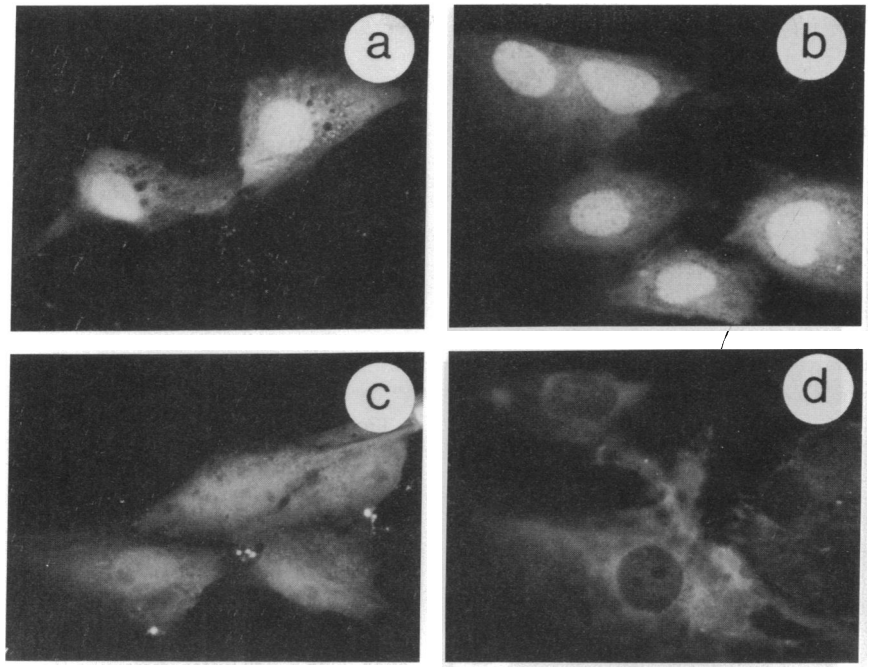

FIG. 2. Nonmyristoylated forms of the catalytic subunit are translocated to the nucleus while type I regulatory subunit is maintained in the cytoplasm. Rat-2 fibroblasts were injected with purified murine catalytic subunit isolated from *E. coli* (26) (*a*), recombinant catalytic subunit lacking a myristoylation site (24) (*b*), chicken ovalbumin (*c*), or bovine type I regulatory subunit (*d*). Cells were fixed 120 min after injection. Murine recombinant catalytic subunit was injected at 125 μM, mutant catalytic subunit at 75 μM, and ovalbumin at 126 μM. The same distribution of murine catalytic subunit was observed when injected at 35 μM. (×800.)

labeled regulatory subunits. The cytoplasmic fluorescence pattern observed for holoenzyme containing labeled catalytic subunit (Fig. 3*a*) was altered by the addition of cAMP. When this holoenzyme was dissociated with Br^8cAMP either *in vitro* prior to injection (Fig. 3*b*) or in the cells after injection (data not shown), the labeled catalytic subunit was translocated to the nucleus. Although under these conditions somewhat more cytoplasmic staining was observed than for injection of free catalytic subunit, the nuclear localization was nevertheless unambiguous. Holoenzyme containing fluorescently labeled regulatory subunit and unlabeled catalytic subunit also localized exclusively in the cytoplasm (Fig. 3*c*). However, the localization remained cytoplasmic when this preparation of holoenzyme was dissociated *in vitro* prior to injection (Fig. 3*d*) or in the cells after injection (data not shown).

The distribution of free catalytic and regulatory subunits and reassociated holoenzyme was analyzed in living cells, as cell fixation can lead to protein loss or redistribution. For these experiments the labeled molecules were visualized without prior cell fixation. The distribution of individual subunits and holoenzyme in living cells reflected the results observed with fixed cells described above: the catalytic subunit was transported from its injection site in the cytoplasm to the nucleus (Fig. 4*a*), whereas free regulatory subunit and reconstituted holoenzyme preparations were mainly cytoplasmic (Fig. 4 *b*, *c*, and *d*).

DISCUSSION

Many studies have examined the cellular distribution of cAPK, with inconsistent results (16–22). We examined the

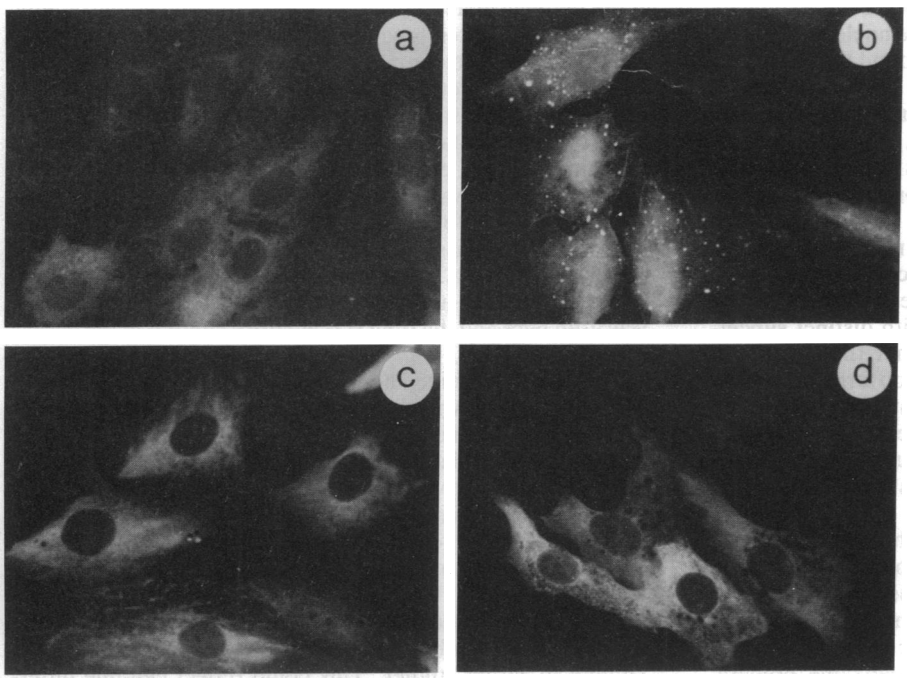

FIG. 3. Type 1 holoenzyme is cytoplasmically localized, but upon dissociation the catalytic subunit is transported to the nucleus while the type I regulatory subunit is maintained in the cytoplasm. Reconstituted holoenzyme containing either fluorescently labeled catalytic subunit (*a*) or type I regulatory subunit (*c*) was injected. In parallel, holoenzyme preparations containing labeled catalytic subunit (*b*) or labeled regulatory subunit (*d*) were incubated with a 5-fold molar excess of 8-bromo-cAMP (Br^8cAMP) *in vitro*, injected, and observed 45 min later. Similar results were obtained when holoenzyme was dissociated *in vivo* by treating injected cells with 1 mM Br^8cAMP. The punctate staining observed in *b* was especially prominent with this preparation of holoenzyme but was occasionally observed with other labeled proteins and probably represents vessicular compartmentalization of fluorescent breakdown products. Holoenzyme was injected at 8–90 μM. In all cases, the cellular distribution was found to be the same. (×800.)

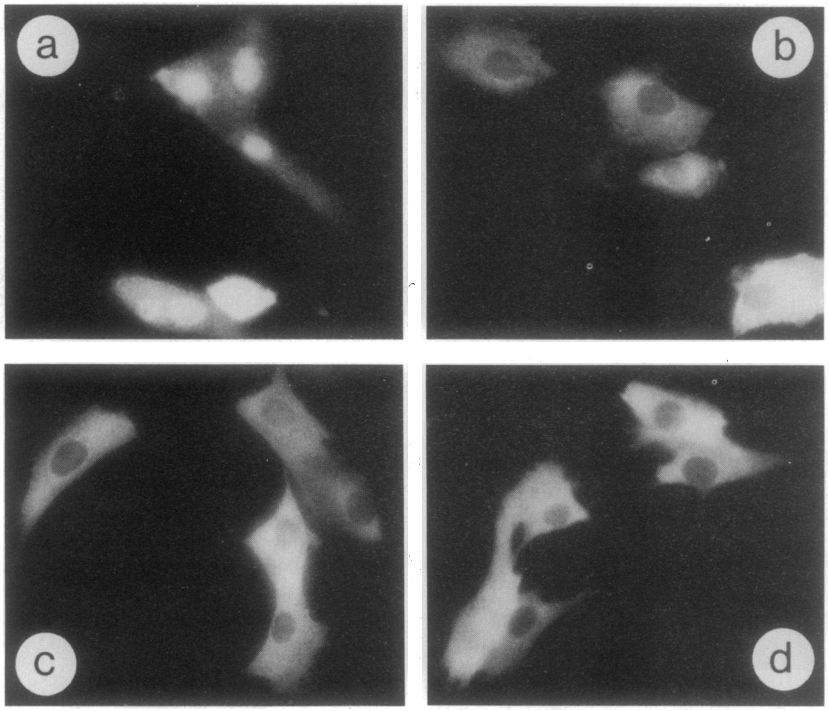

FIG. 4. Free catalytic subunit, but not type I regulatory subunit or holoenzyme, is transported to the nucleus in living fibroblasts. Rat fibroblasts were injected with murine recombinant catalytic subunit (*a*), type I regulatory subunit (*b*), or reconstituted holoenzyme containing labeled catalytic (*c*) or regulatory (*d*) subunits. Cells were observed 60 min after injection, without prior fixation, with a ×40 water-immersion lens. (×425.)

fate of holoenzyme and catalytic and regulatory subunits independently in the same cell type by labeling the purified proteins with FITC and injecting them into living fibroblasts. This approach enabled the analysis of the intracellular distribution of free catalytic and regulatory subunits of the type I holoenzyme and the dynamics of holoenzyme dissociation in living cells.

When injected alone or when dissociated from the regulatory subunit, the catalytic subunit of cAPK is transported to the nucleus within minutes following its introduction into the cytoplasm. Although, in principle, the catalytic subunit is small enough to freely diffuse through nuclear pores (reviewed in ref. 31), the results described here are consistent with either specific transport of the catalytic subunit to the nucleus or its specific retention within the nucleus. Since fluorescently labeled ovalbumin, a protein having a similar molecular weight, is found throughout the cell following microinjection, the specific nuclear fluorescence exhibited by the catalytic subunit is not due to simple nuclear diffusion of molecules in this size range. We have found that catalytic subunit purified from porcine heart (32) and recombinant protein purified from *E. coli* are both found in the nucleus after cytoplasmic injection. In addition, mutant catalytic subunit lacking a myristoylation site (25) is transported to the nucleus, indicating that nuclear localization does not depend solely upon myristoylation.

Unlike free catalytic subunit, the type I regulatory subunit of cAPK exhibits cytoplasmic fluorescence following injection although its distribution is more selective than that of ovalbumin and may reflect association with distinct subcellular structures. *In vivo*, it is thought that the regulatory subunit exists as a dimer of ≈86 kDa (2), too large for simple diffusion between the cellular compartments. In experiments where holoenzyme consisting of fluorescently labeled regulatory subunits and unlabeled catalytic subunits was dissociated prior to injection, the regulatory subunit was found exclusively in the cytoplasm.

In cells not treated with cAMP, type I holoenzyme exists predominantly in the cytoplasm and shows a distribution similar to the free regulatory subunit. Thus, if nuclear localization signals exist in the catalytic subunit, it is likely that they are not functional when the catalytic subunit is part of a holoenzyme complex. Dissociated holoenzyme preparations containing labeled catalytic subunits exhibit primarily nuclear fluorescence, whereas holoenzyme containing labeled regulatory subunits continues to exhibit only cytoplasmic fluorescence. Similar results were obtained when holoenzyme was injected and the cells treated with Br^8cAMP to dissociate the injected holoenzyme *in vivo*. These data all support the conclusion that holoenzyme exists in the cytoplasm. Upon dissociation by increased intracellular cAMP, a portion of the catalytic subunit migrates to the nucleus while the regulatory subunit remains in the cytoplasm.

Alternative strategies have been used to analyze the distribution of catalytic and regulatory subunits by using fluorescein-labeled heat-stable protein kinase inhibitor and fluorescein-conjugated catalytic subunit as probes for staining fixed cells. The results reported here differ from these in certain respects. Upon elevation of cAMP levels in hepatoma cells, catalytic and regulatory subunits (21, 22) appeared first in the cytoplasm and nucleolus and in the nucleoplasm only at later times. Taken together, these results suggest a nucleolar reservoir of holoenzyme. In the present study, nucleolar localization was not detected with either subunit or holoenzyme. Nucleolar staining may have been obscured by the intense nuclear staining observed with injected catalytic subunit; however, even at early time points selective nucleolar localization was not observed. Nucleolar fluorescence was not detected following microinjection of labeled regulatory subunit or dissociated holoenzyme containing labeled regulatory subunits. This may reflect differences in the type I and II regulatory subunit, since the type I regulatory subunit was used here and the type II regulatory subunit in the earlier reports.

The results described here are in close agreement with two earlier reports in which the subcellular distribution of type II cAPK was examined (17, 18). By using well-characterized monospecific antibodies, both the catalytic and the type II regulatory subunit were localized to the Golgi complex and found to be associated with centrosomes (18). Upon activation of adenylate cyclase with forskolin, the catalytic subunit was translocated to the nucleus while the type II regulatory subunit remained Golgi-associated (17). The results described here indicate that the type I regulatory subunit differs in subcellular distribution from that described for the type II subunit in earlier studies. This could reflect cell-line differ-

ences or may indicate that the type I and type II kinases may be differentially localized in the cell.

The finding that the catalytic subunit localizes to the nucleus is consistent with its ability to stimulate transcription from cAMP-inducible promoters (14, 15). In the rat fibroblasts used for these studies, injection of the catalytic subunit, but not type I regulatory subunit, is sufficient to induce the expression of a stably transfected cAMP-inducible marker gene (data not shown). Thus, in this cell line, the catalytic subunit is found in the nucleus and is capable of transcriptional activation. These results suggest that microinjected catalytic subunit is biologically active and responds in a fashion similar to endogenous subunits. At least one of the apparent physiological substrates of the catalytic subunit is the nuclear protein CREB, the cAMP response element-binding protein (33). CREB has been shown to transactivate cAMP-inducible promoters and to require phosphorylation by cAPK for transcriptional induction (34).

The cytoplasmic location of type I regulatory subunit, together with the cytoplasmic and nuclear localization of the active catalytic subunit, suggests that one biological function of the regulatory subunit is to serve as a "cytoplasmic anchor" for the catalytic subunit (35), sequestering it in the cytoplasm in the absence of cyclic nucleotide. Physiologically, increases in intracellular cAMP are associated with numerous cellular responses, including secretion and cytoskeletal changes that might require phosphorylation of cytoplasmic or membrane substrates (23). In the absence of cAMP, the catalytic subunit is thus sequestered in the cytoplasm in close proximity to these potential substrates. These results also provide a possible explanation for the unique mechanism associated with the activation of cAPK. Of all of the protein kinases, only cAPK activation involves the dissociation of dissimilar subunits (24). In general, this is not a mechanism that is commonly invoked for cytoplasmic enzymes.

The approach described here could be used to investigate the intracellular distribution of the type II regulatory subunit as well as the effect of various mutations upon intracellular distribution of catalytic subunits. Through this approach, it should be possible to determine whether catalytic activity is a requirement for nuclear translocation, whether specific targetting sequences are required for nuclear localization of a highly basic protein such as the catalytic subunit, and the effects of introduction of a cytoplasmically sequestered catalytic subunit on cAMP-inducible gene expression. Finally, it will be important to determine whether different types of regulatory subunit behave differently with regard to intracellular localization.

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